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Emergence of cefiderocol resistance during ceftazidime/avibactam treatment caused by a large genomic deletion, including *ampD* and *piuCD* genes, in *Pseudomonas aeruginosa*

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ABSTRACT We report the emergence of cefiderocol resistance during the treatment of a ST312 *Pseudomonas aeruginosa* respiratory infection with ceftazidime/avibactam. whole genome sequencing (WGS) revealed that resistance was caused by a large genomic deletion, including PiuDC (iron transport system) and AmpD (*ampC* negative regulator), driven by the integration of phage DNA. Thus, our findings alert that this type of deletion could be an efficient (two mechanisms in one step) specific cefiderocol resistance mechanism that might occur nonspecifically upon treatment with β-lactams that select for AmpC overexpression.

KEYWORDS *Pseudomonas aeruginosa*, drug resistance evolution, cefiderocol, ceftazidime-avibactam, iron transport

Pseudomonas aeruginosa, a ubiquitous opportunistic pathogen considered one of the paradigms of antimicrobial resistance, is among the main causes of hospital-acquired and chronic infections associated with high morbidity and mortality (1). This growing threat results from the extraordinary capacity of this pathogen for developing resistance through chromosomal mutations and from the increasing prevalence of transferable resistance determinants, particularly those encoding carbapenemases or extended spectrum beta-lactamases (ESBLs) (2, 3), leading to frequent multidrug resistance (MDR) or even extensively drug resistance (XDR) (4) and difficult-to-treat (DTR) (5) phenotypes. The recent introduction of novel β-lactam/β-lactamase inhibitor combinations (such as ceftolozane/tazobactam, ceftazidime/avibactam, or imipenem/relebactam) and the siderophore-cephalosporin cefiderocol has helped to mitigate, to some extent, the problem of XDR/DTR *P. aeruginosa* (6–8). Indeed, these agents show increased stability against the classic *P. aeruginosa* chromosomally-encoded β-lactam resistance mechanisms, such as the overexpression of the chromosomal β-lactamase AmpC and efflux pumps or OprD inactivation. However, they are not exempt from resistance development through emerging mutational mechanisms, such as the modification of the catalytic center of AmpC or the modification of efflux pump substrate specificity, as evidenced right upon their introduction into clinical practice (9–12). However, cefiderocol resistance additionally requires, specifically and differentially from other novel β-lactams, mutations in iron transport systems such as PiuA/D-PiuC or PirA-PirR (13).

Here we report the emergence of *in vivo* cefiderocol resistance development in *P. aeruginosa* caused by a large genomic deletion, including the PiuDC region, in the absence of cefiderocol treatment. This deletion was likely selected by ceftazidime/avibactam treatment since it also included the *ampC* regulator AmpD, which is located close to the PiuDC system.

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A COPD patient was admitted to the intensive care unit of a Spanish hospital (H. Clínico, Valencia) because of a subarachnoid hemorrhage and was intubated. At 48 h, signs of ventilator-associated pneumonia were evident, and *Staphylococcus aureus*, *Enterobacter cloacae*, and *Serratia marcescens* were isolated from respiratory samples. The patient received piperacillin/tazobactam (3 days), meropenem (11 days), and linezolid (12 days). On day 14, an XDR *P. aeruginosa* strain (piperacillin/tazobactam, ceftazidime, cefepime, imipenem, and ciprofloxacin R, meropenem I, ceftazidime/avibactam, and ceftolozane/tazobactam S) was isolated from a respiratory sample, and treatment was initiated with ceftazidime/avibactam for 7 days. Strain PAHCV-190D5 was isolated from a day 28 control (7 days after ceftazidime/avibactam treatment ended) tracheal aspirate (>10^6 CFU/mL). Initial susceptibility testing (MicroScan commercial broth microdilution panel NM63) revealed ceftazidime/avibactam resistance development, and therefore the strain was subjected to extended susceptibility testing through broth microdilution following European Committtee on Antimicrobial Susceptibility Testing (EUCAST) guidelines and breakpoints (v.13.1), using iron-depleted cation-adjusted Muller-Hinton in the case of cefiderocol. Since the parent ceftazidime/avibactam susceptible strain was, unfortunately, not available for comparison, two isolates recovered from two cystic fibrosis (CF) patients showing the same ST (see below), obtained from the collection of hospital Son Espases (Palma de Mallorca, Spain), were characterized in parallel.

As shown in Table 1, strain PAHCV-190D5 showed an XDR/DTR profile and was resistant to all tested β-lactams with the exception of ceftolozane/tazobactam, which showed borderline susceptibility (4 mg/L). Remarkably, the strain was not only resistant to ceftazidime/avibactam but also to imipenem/relebactam, and most notably, cefiderocol, according to EUCAST breakpoints. In contrast, the two control strains from the same clone were susceptible to most β-lactams, including all those recently introduced.

WGS (Miseq, Illumina) and resistome (horizontally acquired and mutational) analysis of the three strains was performed as previously described (15, 16), and results are also shown in Table 1. The strain belonged to ST312, which is not considered a widely disseminated high-risk clone (17), but that has been recently described as associated with plasmid-mediated KPC-2 production in Brazil (18). None of the sequenced ST312 showed acquired resistance determinants, but strain PAHCV-190D5, as compared with the other two isolates, showed a vast mutational resistome responsible for the XDR/DTR phenotype, which included a GyrA D87Y mutation (fluoroquinolone resistance), a premature stop codon in OprD (carbapenemen resistance), an inactivating mutation in the MexAB-OprM efflux pump negative regulator *mexR* (broad resistance, including most β-lactam), and a large (37282 nt using PAO1 as template and 39046 nt using ST312 as template) genomic deletion, including the gene coding the *ampC* regulator AmpD, and, particularly noteworthy, the iron transport system PiuDC. The three ST312 isolates harbored the same AmpC (*Pseudomonas* derived cephalosporinase, PDC) variant (PDC-68), showing G27D, T105A, Q155R, V205L, V356I, and G391A amino acid replacements respect PAO1 (PDC-1). Since the same PDC variant was documented in the susceptible and resistant isolates, its direct impact on cefiderocol resistance should be marginal, if any.

In order to assess the role of the deleted genes in cefiderocol resistance, MICs were determined for *piuA* (*piuD* homolog), *piuC*, and *ampD* knockout mutants obtained from an available PAO1 transposon mutant library (14) and results are shown in Table 1. Whereas the inactivation of *ampD* (and the consequent *ampC* overexpression) by itself did not significantly affect cefiderocol MICs, the inactivation of both *piuA* and *piuC* increased MICs by 4–5 twofold dilutions, as previously evidenced (13).

The complete deleted region is represented in Fig. 1. Moreover, the deletion was caused by the integration of a 37-kb phage element nearly identical to AIIMS-Pa-B1 from the *Caudoviricetes* class and the *Casadabanvirus* genus. This phage was first detected in the Ganga river (India) (NC_061436) and detected integrated in several *P. aeruginosa* strains from the USA (CP104695, ST823), China (CP054790, ST244), or the UK (CP104254,

TABLE 1 Characterization of the three ST312 clinical isolates and PAO1 *ampD*, *piuA*, and *piuC* mutants

	Relative mRNA level[a]				MIC (µg/mL)[b]													
Strain	ampC	mexB	mexY	Mutational resistome	TIC R > 16	TZP R > 16	ATM R > 16	TAZ R > 8	FEP R > 8	AMK R > 16	TOB R > 2	IPM R > 4	MEM R > 8	CST R > 4	CIP R > 0.5	C/T R > 4	C/A R > 8	I/R R > 2	FDC R > 2
PAO1	1	1	1	–	16	≤4	4	1–2	2	≤2	0.5	2	1	1	≤0.12	≤0.5	1	0.25	0.06–0.12
PAHCV-190D5	176.4 ± 35.3	21.4 ± 0.3	1.84 ± 0.3	*mexR* (aa68Δ1), *oprD* (W339X), *parS* (A29V), *gyrA* (D87Y), Δ5033480–5070760 (PA4497–PA4526 including both *ampDE* and *piuCD*)	256	256	64–128	64	32	≤2	0.5	32	32	2	2–4	4	16–32	4	4–8
FQSE31	0.4 ± 0.02	1.7 ± 0.03	1.0 ± 0.07	*parS* (A29V)	16–32	≤4	4	2	1–2	2–4	0.5–1	2	≤0.5	2	0.12–0.25	≤0.5	2	0.25	0.12
FQSE34	1 ± 0.2	2.4 ± 0.2	14.8 ± 0.5	*parS* (A29V), *mexZ* (aa62Δ4, Δ2798520–2840792 (PA2480–PA2520 including *mexST-mexEF-oprN*)	16–32	8	8	2	8–16	16	1	2	2	1	0.25	1	2	0.25	0.12
PAOUWΔampD	21.2 ± 0.4	ND	ND	Δ*ampD*	64	128	8–16	16	8–16	≤2	0.5	2	2	2	≤0.12	1	2	0.12	0.06
PAOUWΔpiuA	ND	ND	ND	Δ*piuA*	≤8	≤4	2–4	1–2	≤1	≤2	0.5	1–2	≤0.5	1–2	≤0.12	≤0.5	1–2	0.25	2
PAOUWΔpiuC	ND	ND	ND	Δ*piuC*	≤8	≤4	2–4	1–2	≤1	≤2	≤0.25	1–2	≤0.5	1–2	≤0.12	0.5–1	1–2	0.25	2

[a]Relative mRNA levels *for ampC*, *mexB*, and *mexY* respect those of wild-type PAO1 determined by real-time RT-PCR as described previously (14).
[b]Broth microdilution MICs were determined in duplicate. Both MIC values obtained are shown when they are not identical. EUCAST v.13.1 resistance breakpoints are indicated for each drug. TIC, ticarcillin (range concentrations tested, 8–512 µg/mL); TZP, piperacillin/tazobactam (4–256 µg/mL); ATM, aztreonam (2–128 µg/mL); TAZ, ceftazidime (1–64 µg/mL); FEP, cefepime (1–64 µg/mL); AMK, amikacin (2–128 µg/mL); TOB, tobramycin (0.25–32 µg/mL); IPM, imipenem (0.5–64 µg/mL); MEM, meropenem (0.5–64 µg/mL); CST, colistin (0.5–16 µg/mL); CIP, ciprofloxacin (0.12–16 µg/mL); C/T, ceftolozane/tazobactam (0.5–32 µg/mL); C/A, ceftazidime/avibactam (0.5–32 µg/mL); I/R, imipenem/relebactam (0.12–64 µg/mL); and FDC, cefiderocol (0.03–16 µg/mL).

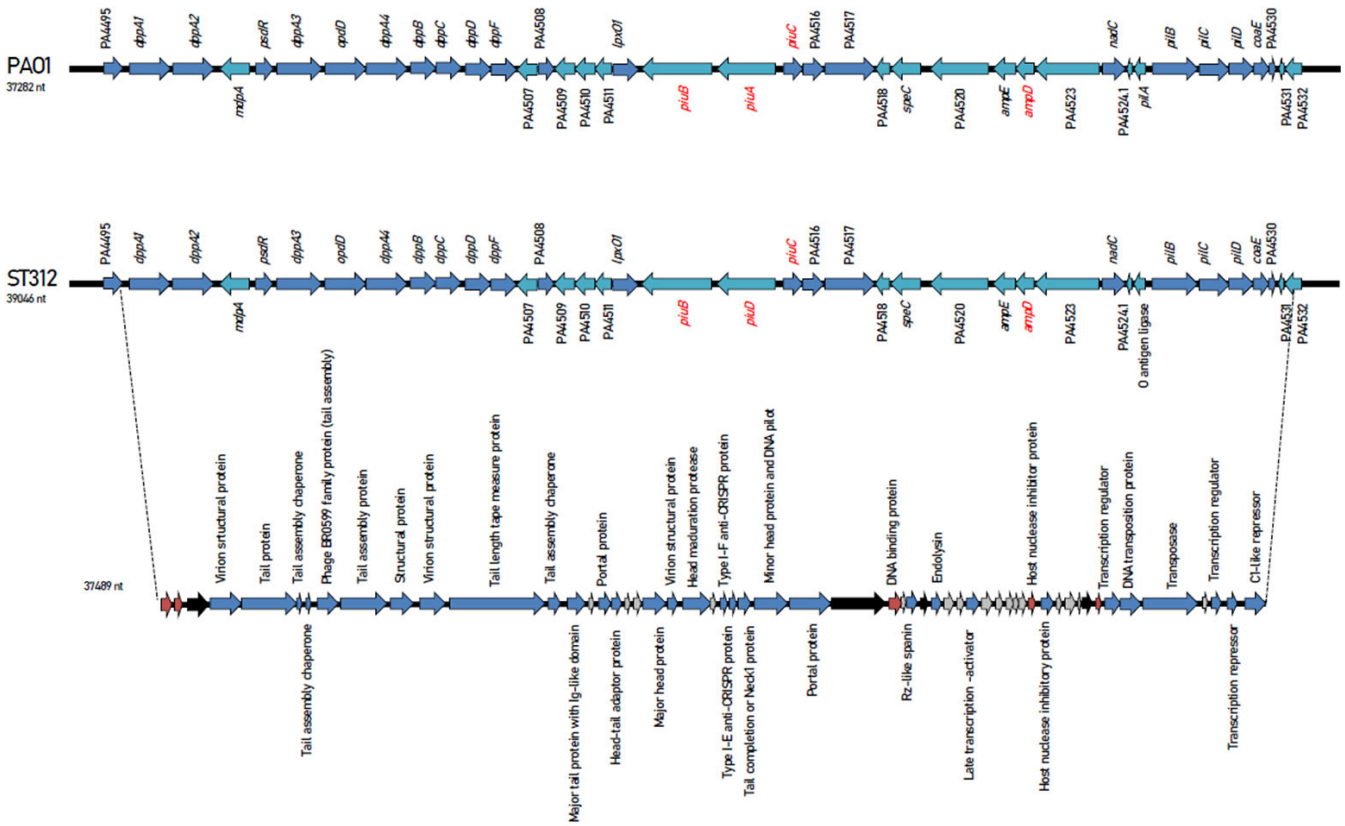

FIG 1 Schematic representation of the genomic region deleted In the cefiderocol-resistant PAHCV-190D5 clinical strain compared to PAO1 and ST312 CF isolates and the genome structure of the inserted phage. Blue arrows indicate functional genes. Red arrows indicate uncompleted genes, gray arrows indicate genes encoding for hypothetical proteins, and black ones indicate genes not present in the related *Pseudomonas aeruginosa* phage AIIMS-Pa-B1 (NC_061436).

ST316). These *P. aeruginosa* strains belong to different STs, and phages were integrated into different genomic regions, which indicates that phage AIIMS-Pa-B1 shows a broad host range.

In order to confirm the effect of the detected mutations in the regulators *mexR*, *mexZ,* and *ampD*, the expression of *mexB*, *mexY,* and *ampC* was determined by real-time RT-PCR as previously described (19). As shown in Table 1, strain PAHCV-190D5 showed greatly increased *mexB* and *ampC* expression compared to wild-type PAO1 and the two control ST312 strains, confirming the effect of the *mexR* mutation and *ampD* deletion, respectively. Thus, OprD inactivation + MexAB-OprM overexpression likely explains imipenem/relebactam resistance, whereas MexAB-OprM overexpression + AmpC overexpression likely explains ceftazidime/avibactam resistance in this strain. In contrast, one of the control strains (FQSE-34) showed increased *mexY* expression and high cefepime and amikacin MICs, confirming the expected effect of the documented *mexZ* mutation. The effect of the large genomic deletion (including the *mexST-mexEF-oprN* region) detected in this same strain on the resistance phenotype is less obvious. Likewise, despite the fact that the three studied strains belonged to ST312, cgMLST analysis, performed with chewBBACA version 3.2.0 (20), revealed 246-347 (of 5,676) allelic differences, indicating that they are not closely related.

Summing up, we report the emergence of cefiderocol resistance development in *P. aeruginosa* in the absence of cefiderocol treatment, caused by a large genomic deletion, including the PiuDC region. This deletion was likely selected by ceftazidime/avibactam treatment, since it also included the *ampC* regulator AmpD and was driven by the integration of a phage DNA. While the investigation of the specific interplay between the integration of the phage and the genomic deletion falls outside of the scope of this work,

our findings alert us that this type of deletion could be an efficient (two mechanisms in one step) specific cefiderocol resistance mechanism that might occur nonspecifically upon treatment with β-lactams that select for AmpC overexpression.

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María A. Gomis-Font, Data curation, Investigation, Writing – review and editing | María A. Clari, Formal analysis, Methodology, Writing – review and editing | David Navarro, Formal analysis, Methodology, Writing – review and editing | Antonio Oliver, Conceptualization, Formal analysis, Funding acquisition, Supervision, Validation, Writing – original draft.

DATA AVAILABILITY

Sequence files have been deposited in the European Nucleotide Archive under study number PRJEB65984.

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